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Published in:
British Journal of Cancer

DOI:
[10.1038/bjc.2014.420](https://doi.org/10.1038/bjc.2014.420)

Publication date:
2014

Licence:
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Document Version
Publisher's PDF, also known as Version of record

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

El-Mokadem, I., Fitzpatrick, J., Bondad, J., Rauchhaus, P., Cunningham, J., Pratt, N., Fleming, S., & Nabi, G. (2014). Chromosome 9p deletion in clear cell renal cell carcinoma predicts recurrence and survival following surgery. *British Journal of Cancer*, 111(7), 1381-1390. <https://doi.org/10.1038/bjc.2014.420>

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Keywords: renal cell carcinoma; prognosis; chromosome 9p; fluorescence *in situ* hybridisation; clear cell; biomarker

Chromosome 9p deletion in clear cell renal cell carcinoma predicts recurrence and survival following surgery

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Background: Wider clinical applications of 9p status in clear cell renal cell carcinoma (ccRCC) are limited owing to the lack of validation and consensus for interphase fluorescent *in situ* hybridisation (I-FISH) scoring technique. The aim of this study was to analytically validate the applicability of I-FISH in assessing 9p deletion in ccRCC and to clinically assess its long-term prognostic impact following surgical excision of ccRCC.

Methods: Tissue microarrays were constructed from 108 renal cell carcinoma (RCC) tumour paraffin blocks. Interphase fluorescent *in situ* hybridisation analysis was undertaken based on preset criteria by two independent observers to assess interobserver variability. 9p status in ccRCC tumours was determined and correlated to clinicopathological variables, recurrence-free survival and disease-specific survival.

Results: There were 80 ccRCCs with valid 9p scoring and a median follow-up of 95 months. Kappa statistic for interobserver variability was 0.71 (good agreement). 9p deletion was detected in 44% of ccRCCs. 9p loss was associated with higher stage, larger tumours, necrosis, microvascular and renal vein invasion, and higher SSIGN (stage, size, grade and necrosis) score. Patients with 9p-deleted ccRCC were at a higher risk of recurrence ($P=0.008$) and RCC-specific mortality ($P=0.001$). On multivariate analysis, 9p deletion was an independent predictor of recurrence (hazard ratio 4.323; $P=0.021$) and RCC-specific mortality (hazard ratio 4.603; $P=0.007$). The predictive accuracy of SSIGN score improved from 87.7% to 93.1% by integrating 9p status to the model ($P=0.001$).

Conclusions: Loss of 9p is associated with aggressive ccRCC and worse prognosis in patients following surgery. Our findings independently confirm the findings of previous reports relying on I-FISH to detect 9p (CDKN2A) deletion.

The predictive role of the detection of chromosomal and gene copy number variation (CNV) in renal cell carcinoma (RCC) has been explored in several studies (Gunawan *et al*, 2001; Klatte *et al*, 2009). Deletion of the short arm of chromosome 9 (9p) has been confirmed, in many studies, to be one of the most frequent non-random chromosomal CNVs in clear cell RCC (ccRCC) (Strefford *et al*, 2005; Matsuda *et al*, 2008; Beroukheim *et al*, 2009; Dalglish *et al*, 2010). Moreover, accumulating evidence from other cancers

indicates frequent 9p deletions to be seen in high-grade invasive and aggressive cancers (Sidransky, 1995; Okami *et al*, 1997; Ellsworth *et al*, 2008). Potential genes on chromosome 9p have been implicated in ccRCC progression. The cyclin-dependent kinase inhibitor (CDKN2A) gene is a tumour suppressor gene, located on 9p21, encodes two proteins (*p16INK4* and *p14ARF*) that regulate two critical cell cycle regulatory pathways: the p53 pathway and the Rb pathway (Harris and Levine, 2005). Thus, the inactivation of

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Received 11 April 2014; revised 25 June 2014; accepted 1 July 2014; published online 19 August 2014

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CDKN2A gene via 9p21 deletion, leading to low expression of these regulatory proteins, may be an important biomarker of cancer aggressiveness (Kawada *et al*, 2001a; Ikurowo *et al*, 2007). Also, the low level of expression of carbonic anhydrase IX (CAIX) gene located on locus 9p13 has been associated with shorter survival and poor response in patients on immunotherapy for metastatic lesions (Bui *et al*, 2003; Atkins *et al*, 2005; Sandlund *et al*, 2007; Patard *et al*, 2008). Thus, established loss of 9p in RCC could act as a marker of tumour aggressiveness and could be responsible for progression (Cairns *et al*, 1995; Kinoshita *et al*, 1995; Schullerus *et al*, 1997; Junker *et al*, 2003; Klatte *et al*, 2009; Dagher *et al*, 2013).

Recently, two studies, using interphase fluorescence *in situ* hybridisation (I-FISH) as the main detection technique, have concluded that loss of 9p in ccRCC was an independent prognostic factor besides other known pathological parameters (Brunelli *et al*, 2008; La Rochelle *et al*, 2010). Although the findings from these studies were promising, they were both of limited clinical applicability owing to lack of standardisation of I-FISH scoring, lack of reporting on degree of interobserver variability and a modest follow-up period. La Rochelle *et al* (2010) performed I-FISH analysis on a cohort of 316 patients but the cut-off level for 9p deletion was arbitrary, which could have possibly resulted in underestimation of CNVs.

As I-FISH can be performed on both fresh and archived formalin-fixed paraffin-embedded (FFPE) tumour tissue, it has been used widely on tissue microarrays (TMAs) that allow the assessment of many tumours in one experiment, in comparison to conventional cytogenetics that can only be performed on individual cases from fresh tissue after culture. Also, I-FISH assesses CNVs at a cellular level, in contrast to other techniques, such as array comparative genomic hybridisation and microsatellite analysis based on mass DNA extraction, which can conceal CNVs and intratumour cellular genetic complexity and heterogeneity (Bayani and Squire, 2007).

On the other hand, standardisation of I-FISH scoring techniques and reaching a consensus on how to determine the threshold level are required before the technique can be applied in translational renal oncology (Junker *et al*, 2013). This is due to the fact that the scoring process can be influenced by fading signals, sectioning of paraffin blocks and quality of tissue preserved, which can result in interobserver disagreement.

Our study aims to assess the prognostic effect of 9p deletion in ccRCC subtype. We hypothesised that patients with 9p-deleted ccRCC tumours detected by I-FISH do not carry a worse prognosis (H_0). A set of objectives was identified to test our hypothesis: (a) to analytically validate I-FISH scoring technique for assessing 9p status in renal cancer, (b) to assess the degree of interobserver variability in scoring 9p status, (c) to assess the correlation between 9p status and other established clinicopathological prognostic variables and finally, (d) to assess the impact of 9p loss in ccRCC tumours on recurrence-free survival (RFS) and disease-specific survival (DSS) in a cohort of patients with at least 8 years of follow-up.

MATERIALS AND METHODS

Identification of cohort. The urology department database was searched for consecutive patients who underwent radical nephrectomy for RCC between January 2001 and December 2005 without any exclusion criteria. The period was chosen to allow a sufficient follow-up period of at least 8 years for each patient. Ethical approval (Ref. 12/ES/0083) was obtained from the Tayside Research and Ethics committee. A single pathologist (SF) with special interest in renal cancer, blinded to clinical outcomes, reviewed all the samples using pathology number identifier for each patient.

Tissue microarrays. Haematoxylin and eosin (H&E) slides from all the archived FFPE blocks were reviewed for representative areas in RCC tumours. Tissue microarrays were constructed as described in previous publication from our institution (Hadad *et al*, 2009). Hundred and twenty six FFPE blocks from 108 tumours, identified from the pathology department archives, were used to construct four tumour TMAs. Sixteen tumours were represented by two blocks and one tumour was represented by three blocks. These extra blocks served as internal control to assess the effect of tumour heterogeneity without the knowledge of the scoring observer. One TMA of normal renal tissue from same cases was prepared to serve as a negative control.

Briefly the technique is described here. Tissue microarrays were constructed using Beecher arraying instrument (Beecher Instruments Inc., Sun Prairie, WI, USA) with the help of TMA Designer 2 software (ALPHELYS, Plaisir, France). The pathologist identified and marked, with a circle on the H&E slide, areas containing only tumour for coring, and avoiding areas of necrosis or normal tissue, before marking the same tumour region on the corresponding formalin-fixed paraffin block. Tissue cores with a diameter of 0.6 mm were punched from the marked tumour regions on paraffin blocks. Cores were then deposited into a master paraffin block and placed 1.2 mm apart from the neighbouring core on the *x* and *y* axes. Sections from the resulting master paraffin block measuring 4 μ m in thickness were then transferred onto glass slides to form a TMA. Each TMA contained a maximum of 240 tumour cores plus two spleen control cores for orientation. Each case was represented at least with six tumour cores.

Interphase fluorescent *in situ* hybridisation. Interphase fluorescent *in situ* hybridisation analysis was performed using the Vysis locus-specific identifier (LSI) *CDKN2A* spectrum red (R)/(CEP 9) spectrum green (G) probes (Abbott Molecular, Des Plaines, IL, USA). The *CDKN2A* probe spans ~222 kb of the 9p21 region across several genes, including p16 (INK4A), p14 (ARF) and p15 (INK4B). CEP 9 is a centromeric probe that hybridises to α -satellite sequences specific to chromosome 9 (9p11-q11).

Tissue microarray slides were baked for 60 min at 60 °C in a dry incubator and then processed in automated Vysis VP-2000 processor (Abbott Molecular) to deparaffinise the slides. The slides were submersed in Xylene basin for 10 min twice, then alcohol 100% basin for 5 min twice before they were left to dry for 2 min. The slides were left in 0.2 N HCL basin for 20 min before they were rinsed in a water chamber for 3 min. The slides were then washed twice in 2 \times SSC basin each for 3 min before they were removed from VP-2000. Next, slides were put through sodium thiocyanate (NaSCN) pretreatment reagent (Abbott Molecular) at 80 °C for 30 min followed by protease solution (0.5 mg ml⁻¹ 0.01 N HCl) (Abbott Molecular) at 37 °C for 50 min. Slides were then put through three sequential 2-min washes with 2 \times SSC and then dehydrated for 2 min each in graded ethanol solutions (70%, 85% and 100%). Once dried, slides were placed in 70% formamide/2 \times SSC at 70 °C in a water bath for 5 min, removed, put through a further graded ethanol series (70%, 85% and 100%) for 1 min each and then allowed to dry.

For each slide, a probe mix was made up by combining 4 μ l *CDKN2A*/CEP 9 probe, 21 μ l LSI/WCP Vysis hybridisation buffer (Abbott Molecular) and 6 μ l dH₂O in a 200 μ l Eppendorf tube. The probe was mixed and denatured by placing the mix in a 70 °C water bath for 5 min. The mix (31 μ l) was then drawn up and pipetted onto a 22 \times 50 mm coverslip. The dried slide was then lowered onto the coverslip, ensuring that the probe mix was evenly distributed and that no air bubbles were present. Slides were then sealed around the edges with rubber cement Fixogum (Kreatech Diagnostics, Leica Microsystems, Milton Keynes, UK) and placed overnight (~12 h) in a humidified hybridisation chamber in a 37 °C incubator.

The following day, cover slips were peeled off. Slides were placed in a hot wash (0.4 \times SSC/0.3% NP-40 (Vysis, Abbott Molecular))

for 2 min at 70 °C in a water bath and then in a room temperature wash ($2 \times$ SSC/0.1% NP-40) for a further 2 min. Once dried, slides were counterstained with 25 μ l DAPI Antifade (Abbott Molecular), mounted onto a fresh coverslip and edges sealed with nail varnish. Slides were stored in covered slide trays at 4 °C before and after scoring.

Interphase fluorescent *in situ* hybridisation interpretation.

Slides were viewed under an Olympus B \times 60 fluorescent microscope (Olympus Corporation, Tokyo, Japan) with filters for red spectrum, green spectrum and DAPI nuclear counterstain. Scoring was undertaken by two independent observers (IEM and JF) blinded to clinical or pathological outcomes. Each observer counted the number of red and green signals in each nucleus using preset criteria. A minimum of 30 intact non-overlapping cells per core, a minimum of three cores per block and a minimum of 100–200 nuclei with good signals per case were required for valid scoring. If these criteria were not met, the scoring was considered invalid. The interpretation of nuclei probe patterns was as follows: 2G2R was interpreted as normal, abnormal patterns included: 2G1R as loss of heterozygosity (LOH), 2G0R as homozygous deletion and 1G1R as monosomy of chromosome 9. Other nuclei patterns were labelled as ‘unclassified’.

The threshold for 9p deletion was determined by calculating the mean percentage (%) of abnormal nuclei from cores on the TMA of normal renal tissue plus four times the standard deviation as previously described in the literature (Cossu-Rocca *et al*, 2006; Brunelli *et al*, 2008). This translated to the following cut-off values for 9p deletion: 18% for LOH and homozygous loss of 9p (2G1R/2G0R), 37% for monosomy (1G1R), and 42% for all abnormal patterns. A tumour was scored 9p deleted if the number of cores with 9p deletion was higher than those with normal 9p status. When the number of cores with and without 9p deletion was equal, we relied on the mean percentage of 9p-deleted nuclei in all cores to decide on deletion. Each observer independently determined the deletion status on the basis of the above criteria (Figure 1). In tumours represented with cores from more than one block, they were considered deleted if the cores from one of the blocks were scored deleted. The level of interobserver agreement was assessed and any scoring disagreement was settled by consensus.

Clinicopathological data. The clinicopathological and follow-up data up to December 2013 for each patient were retrieved retrospectively using a unique 10-digit Community Health Index number and recorded on a predesigned electronic database sheet by an independent researcher (JB), blinded to chromosome 9p status. The following Tayside Health Board electronic resources were searched to retrieve and were cross-validated for clinical and follow-up information:

Hospital records: namely, clinical portal, ICE and In-House Surgical Information System Web and Technology (in-site). These databases represent single, secure, electronic, patient-record sources.

Multidisciplinary board meeting records: all renal cancer cases were discussed in weekly uro-oncology meeting during the study period. The records for each patient were available as an MS Word file under the heading ‘kidney’.

Data including patients’ gender, age at diagnosis and pre-operative staging were retrieved. Pathology slides review and reports were used to identify tumour stage based on 2009 TNM staging (Sobin *et al*, 2009), tumour size, Fuhrman grade, tumour necrosis, pelvicalyceal invasion, renal sinus invasion, microvascular invasion and sarcomatoid features. Each case was assigned a Mayo Clinic SSIGN(stage, size, grade and necrosis) score and stratified into five subcategories (SSIGN score subcategories 0–2, 3–4, 5–6, 7–9 and ≥ 10 , respectively) (Frank *et al*, 2002). Stage, size, grade

and necrosis score has been externally validated in previous studies and established as a reliable prognostic model (Ficarra *et al*, 2006; Fujii *et al*, 2008; Ficarra *et al*, 2009).

Follow-up was calculated from the time of surgery to the last date of assessment or the date of death. Cause of death was determined on the basis of death certificates and correspondence between clinicians and patients’ general practitioners. All patients were followed up on the basis of a standardised departmental protocol based on tumour stage and grade. Death from renal cancer was defined as disease-related mortality. Recurrence was diagnosed if RCC metastasis or renal bed recurrence was detected on cross-sectional imaging and confirmed in a multidisciplinary team meeting record.

Statistical analysis. The number of cores and nuclei scored by each observer was compared. The level of interobserver agreement upon validity of scoring and deletion status per represented block of tumour was assessed using Kappa statistical analysis.

The clinicopathological data were compared based on 9p status. Proportions between categorical variables were compared using Fisher exact and Pearson chi-square tests, as appropriate. The survival time was summarised using median and interquartile range (IQR). Other continuous variables were summarised as means (s.d.), and compared using Student’s *t*-tests or Wilcoxon rank test, as appropriate. The Kaplan–Meier method was used to estimate RFS and DSS based on 9p status and other variables. The log-rank test was used to compare the survival differences between the groups.

A univariate Cox proportional hazard model was used to assess the correlation between prognostic variables and recurrence, and RCC-specific mortality. Multivariate analysis was performed for DSS and RFS after excluding the insignificant variables on univariate analysis. For DSS, two models were generated: one included all the significant variables except SSIGN score, and the other was fit with SSIGN score and all the other significant variables not incorporated in the SSIGN algorithm. A backward selection manner with the likelihood ratio criterion (for entry and removal: $P \leq 0.05$ and $P > 0.1$, respectively) and rank of elimination was used to identify the most significant variables to be entered in a final model for RFS and two final prognostic models for DSS. The predictive accuracy of prognostic models was assessed by employing Concordance index (C-index). Statistical analysis was performed using IBM SPSS version 21 (IBM Corporation), with all tests being two-tailed and $P < 0.05$ considered statistically significant.

RESULTS

There were 89 (82.4%) patients with ccRCC tumours, 13 (12%) with papillary RCCs (pRCCs), 5 (4.6%) with chromophobe RCCs and 1 (1%) with collecting duct carcinoma. Ten tumours (nine ccRCCs and one pRCC) could not be scored due to poor signal, or core loss, or excess connective tissue, or tissue damage or necrosis.

Interobserver agreement of FISH scoring. Out of 756 tumour cores, observer 1 scored 534 (70%) compared with 517 (68%) cores by observer 2 on the basis of the preset criteria. The mean number of cores per tumour block scored by each observer was comparable (4.6 out of 6 vs 4.45 out of 6; $P = 0.055$). Also, the mean number of nuclei per tumour block scored by each observer was comparable (mean 151.4 vs 147.8; $P = 0.645$). Both observers agreed upon 9p status or invalid scoring in 105 out of 126 (83%) tumour blocks with a kappa statistic for interobserver agreement of 0.71 (good agreement; 95% CI 0.60–0.82; $P < 0.0001$). Deletion was detected and agreed upon in 37 tumour blocks with LOH being the predominant deletion pattern ($n = 21$), followed by monosomy ($n = 13$) and homozygous deletion ($n = 3$). Both observers agreed on a no-deletion status and

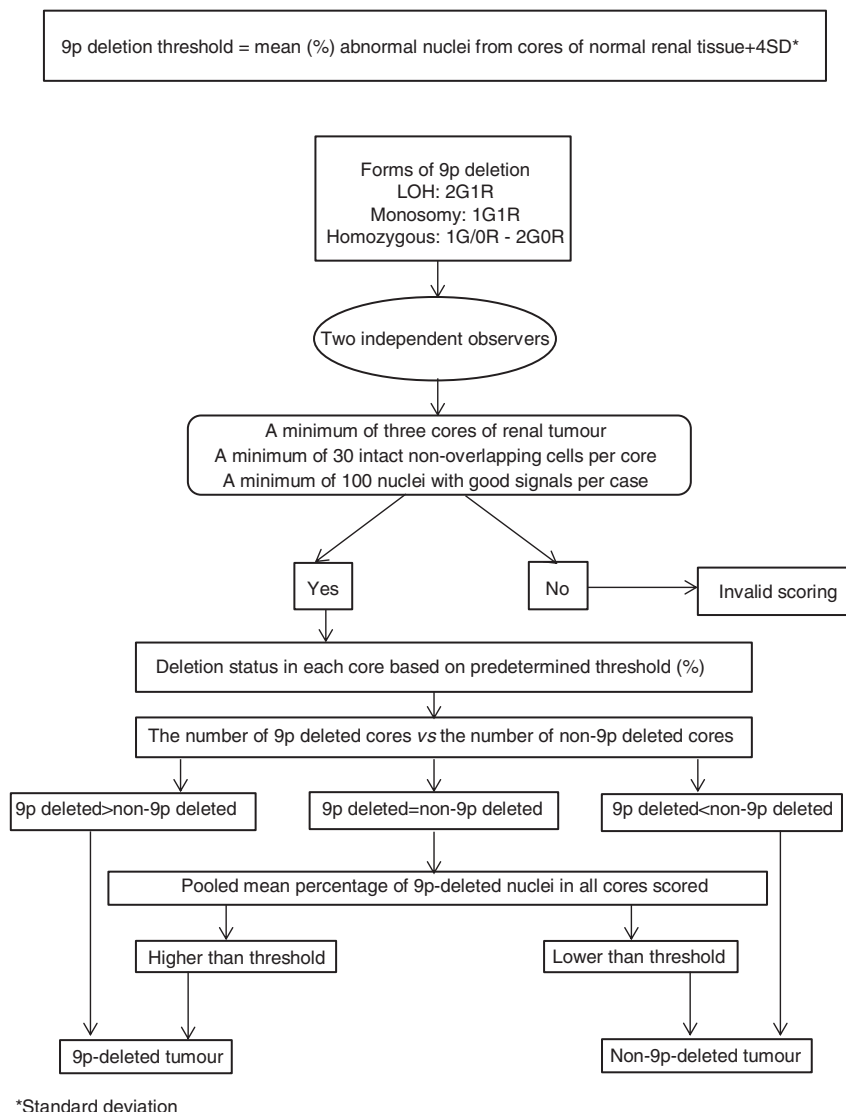


Figure 1. Algorithm for interphase FISH scoring of 9p deletion using the Vysis locus-specific identifier (LSI) CDKN2A spectrum red (R)/ chromosome 9 centromeric-enumeration probe (CEP9) spectrum green (G).

invalid scoring in 58 and 10 blocks, respectively. Two out of the 17 tumours represented by more than one block showed variation for 9p status between the blocks.

The pooled mean percentage of abnormal nuclei, in the agreed upon 9p-deleted tumour blocks, was 52.5% (range 35–97%) and 54% (37–98%) for observer 1 and observer 2, respectively ($P=0.782$). Whereas in the non-9p-deleted tumour blocks, the pooled mean percentage of abnormal nuclei was 23% (range 7–42%) and 25% (6–47%) for observer 1 and observer 2, respectively ($P=0.4$). In the agreed-upon blocks, the detection of 9p deletion increased with the total number of cores scored. 9p deletion was observed in 25% of blocks where three cores were scored compared with almost 50% of blocks where the six representative cores were scored (Figure 2).

Both observers disagreed on scoring 21 tumour blocks. The majority of discordance was due to the invalid scoring by one observer ($n=10$). In the rest of discordant cases ($n=11$), there was a significant difference in the percentage of abnormal nuclei between both observers, which required a slide review to reach a consensus.

We scored 10 whole-tissue sections from tumours on which both observers agreed on 9p status. The five non-deleted tumours showed abnormal nuclei ranging between 11 and 23% (mean 19%)

in whole-tissue section compared with 15 and 30% (mean 20.3%) in the representative cores. On the other hand, the five tumours with 9p deletion showed a range of abnormal nuclei between 66 and 93% (mean 78%) on whole sections compared with a range between 53 and 97% (mean 71%) in the corresponding cores. The overall concordance between whole sections and tissue array cores was 100%.

Clinicopathological parameters. There were 98 out of 108 tumours with valid 9p deletion status and complete pathological and follow-up data for analysis. Only patients with ccRCC tumours ($n=80$), with mean age of 62.96 years (s.d. ± 11.64) and male to female ratio of 5:3, were included in the analysis.

Table 1 summarises the clinicopathological parameters based on chromosome 9p status. 9p deletion was observed in 44% of the tumours (35 out of 80). The mean age of the 9p-deleted group was 65.92 (s.d. ± 10.7) years compared with 60.4 (s.d. ± 11.92) years in the non-9p-deleted group ($P=0.043$).

The mean size of 9p-deleted tumours was 7.7 cm (s.d. ± 3.42) vs 5.6 cm (s.d. ± 2.54) for non-9p-deleted ones ($P=0.002$). Also, 9p loss was significantly associated with higher pathological T-stage ($P=0.021$), presence of tumour necrosis ($P=0.019$),

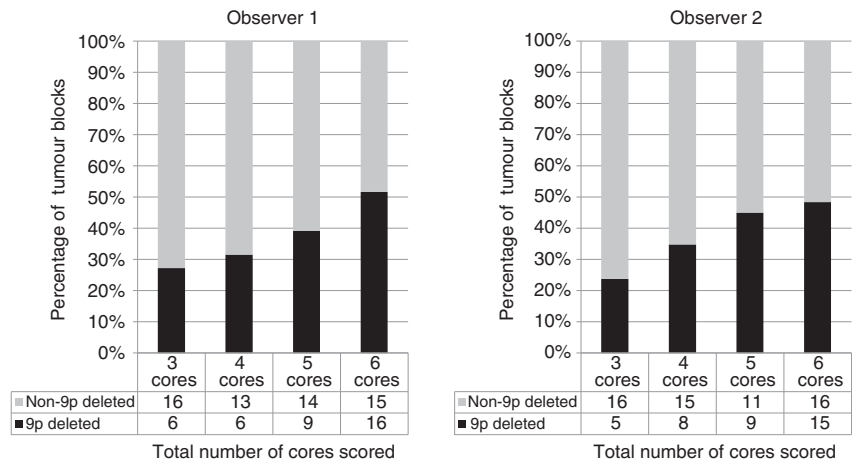


Figure 2. Agreed 9p status in tumour blocks by total number of cores scored.

microvascular invasion ($P=0.032$) and renal vein invasion ($P=0.046$) compared with non-9p-deleted tumours.

Twelve patients (15%) had metastatic disease at diagnosis. Two had regional lymph node metastasis, eight patients had distant metastasis involving typical sites: adrenals, lungs and liver. One patient had metastasis to an unusual site, the bladder wall that was confirmed on resection pathology. Only one patient had both regional lymph node and distant liver metastases. In this cohort, 9p status was not associated with a higher risk of metastasis ($P=0.156$).

In total, there were 30, 8, 17, 16 and 9 patients with 0–2, 3–4, 5–6, 7–9 and ≥ 10 SSIGN scores, respectively. 9p-deleted tumours were associated with higher SSIGN scores ($P=0.033$).

Survival analysis. The median follow-up for patients with ccRCC was 95 months (IQR 40.5–115.5 months). In total, 40 patients (50%) died at the end of the follow-up period, 20 patients (25%) died from advanced renal cancer, whereas 20 patients died from non-RCC-related causes. Nine cases died from a second advanced malignancy including lung ($n=2$), breast ($n=3$), pancreatic ($n=1$), colorectal ($n=2$) and melanoma ($n=1$) with no sign of renal cancer recurrence. One died from sepsis and acute kidney injury on day 8 post nephrectomy. Eleven patients died from other causes including sepsis, cerebrovascular accident, auto-immune hepatitis and severe pancreatitis from gall stones.

The median DSS for tumours with 9p loss was 87 months (IQR 31–112 months) compared with 97 months in non-9p-deleted ones (IQR 53–120 months; $P=0.291$). At 1, 5 and 10 years of follow-up, 15%, 39% and 55% of patients with 9p loss died from ccRCC, respectively, as compared with 0%, 5% and 11% in patients with normal 9p ($P<0.001$) (Figure 3a). Also, tumour size ≥ 5 cm, pathological T3 and T4 stages, Fuhrman grades 3 and 4, presence of metastasis (N + M +), tumour necrosis, microvascular invasion, renal vein invasion, pelvicalyceal invasion, renal sinus invasion and SSIGN scores were all variables associated significantly with cancer-specific mortality on univariate analysis (Table 2).

There were 19 patients with clinically localised small renal masses (SRMs) (cT1a) in this cohort (<4 cm) with a mean size of 2.9 cm (s.d. ± 0.66 cm) with a median follow-up of 107 months (IQR 42–128 months). Two out of the five patients (40%) with 9p-deleted SRMs died from advanced renal cancer. Those two SRMs were found to be invading the renal sinus and perinephric fat and were staged as pT3a. On the other hand, none of the patients with normal 9p status SRMs had a ccRCC-specific death ($P=0.013$).

There were 19 patients with locally advanced non-metastatic ccRCC (pT3N0M0) with a median follow-up of 90 months (IQR 42–105 months). Eight out of 10 patients (80%) with 9p-deleted tumours died from advanced renal cancer compared with 1 out of 9 (11%) with normal 9p status ($P=0.009$; Figure 3c). In this subgroup of patients, 9p loss was the only significant variable on univariate analysis (hazard ratio 10.071; $P=0.026$).

Sixteen out of 68 patients (23.5%) with localised ccRCCs at diagnosis developed recurrence with the median time to recurrence being 35.5 months. 9p deletion, pT-stage, microvascular invasion and age were associated with increased risk of recurrence on univariate analysis in our cohort (Table 2). In the non-9p-deleted tumours, only 11% developed recurrences at 5 and 10 years of follow-up, in contrast to 8%, 34% and 55% of patients with 9p-deleted tumours showing recurrences at 1, 5 and 10 years, respectively ($P=0.004$; Figure 3b). We observed a more aggressive pattern of recurrence in patients with 9p-deleted tumours involving distant organs when first detected; in 11 out of 12 cases disease recurred distantly, whereas 3 out of the 4 patients with non-9p-deleted tumours had the first site of recurrence in the resected renal bed ($P=0.026$).

In multivariate analysis, 9p deletion was an independent prognostic factor in both models for DSS (Table 3). Patients with 9p-deleted tumours had 4.6- and 4-fold higher risk of mortality from advanced renal cancer compared with the non-deleted ones in DSS models 1 and 2, respectively. The inclusion of 9p status in model 1 beside T-stage and state of metastasis improved the predictive accuracy expressed by C-index from 0.858 to 0.911 ($P=0.001$). Adding 9p status to the SSIGN score in model 2 increased the predictive accuracy of the SSIGN score expressed by the C-index from 0.877 to 0.931 ($P=0.001$). In model 3, 9p deletion was an independent predictor of recurrence (hazard ratio 3.863; $P=0.021$) with pT-stage (Table 3).

DISCUSSION

We performed analytic validation of I-FISH in the assessment of 9p deletion in ccRCC in a cohort of patients with the longest reported period of follow-up. We used the same probe reported in the previous studies, and relied on normal renal tissue as a negative control to determine the threshold level for 9p deletion with a good level of interobserver agreement. The use of a negative control to determine the cut-off threshold for deletion makes the scoring method more reliable, objective and reproducible.

Table 1. Clinicopathological parameters by 9p deletion status

Clear cell RCC		Number of cases (n) (%)	
Variables	9p deleted	Non-9p deleted	P-value
Total n	35 (44%)	45 (56%)	
Age at diagnosis (years; mean (s.d.))	65.94 (10.7)	60.4 (11.92)	0.043 ^a
Tumour size (cm, mean (s.d.))	7.7 (3.42)	5.6 (2.54)	0.002 ^a
Gender			
Male	24 (69%)	26 (58%)	0.360
Female	11 (31%)	19 (42%)	
T stage			
pT1a	3 (9%)	15 (33%)	0.021 ^a
pT1b	7 (20%)	11 (24%)	
pT2a	6 (17%)	5 (11%)	
pT2b	2 (6%)	1 (2%)	
pT3a	12 (34%)	13 (29%)	
≥pT3b	5 (14%)	0	
N stage			
N0/x	32 (91%)	45 (100%)	0.080
N1/2	3 (9%)	0	
M stage			
M0	29 (82%)	41 (91%)	0.320
M1	6 (18%)	4 (9%)	
Metastasis			
NOM0	27 (77%)	41 (91%)	0.166
N + M +	8 (23%)	4 (9%)	
Fuhrman grade			
G1/G2	11 (31%)	20 (44%)	0.360
G3/G4	24 (69%)	25 (56%)	
Tumour necrosis			
Present	18 (51%)	11 (24%)	0.019 ^a
Absent	17 (49%)	34 (76%)	
SSIGN score			
0–2	7 (20%)	23 (51%)	0.033 ^a
3–4	5 (14%)	3 (7%)	
5–6	7 (20%)	10 (22%)	
7–9	10 (29%)	6 (13%)	
≥10	6 (17%)	3 (7%)	
Sarcomatoid change			
Present	3 (9%)	3 (7%)	1.0
Absent	32 (91%)	42 (93%)	
Microvascular invasion			
Present	16 (46%)	10 (22%)	0.032 ^a
Absent	19 (54%)	35 (78%)	
Renal vein invasion			
Present	11 (31%)	5 (11%)	0.046 ^a
Absent	24 (69%)	40 (89%)	
Pelvic/lyceal invasion			
Present	6 (17%)	7 (16%)	1.000
Absent	29 (83%)	38 (84%)	

Table 1. (Continued)

Clear cell RCC		Number of cases (n) (%)	
Variables	9p deleted	Non-9p deleted	P-value
Renal sinus invasion			
Present	10 (29%)	6 (13%)	0.102
Absent	25 (71%)	39 (87%)	
Abbreviations: RCC = renal cell carcinoma; SSIGN = stage, size, grade and necrosis.			
^a Statistically significant			

It is known that intratumour heterogeneity and complexity could be underestimated or missed as a result of sampling bias if a small number of biopsy cores are used (Martinez *et al*, 2013). Also, there is a general realisation that biomarker research using TMA may not be sufficient to adequately represent whole-tissue specimens (Eckel-Passow *et al*, 2010). The detection rate of 9p deletion in the current study is 44%, which is higher than that reported in the literature—ranging between 14 and 18% in studies employing I-FISH on TMA (Brunelli *et al*, 2008; La Rochelle *et al*, 2010).

The higher detection rate of 9p deletion could be partly explained by the difference in cut-off threshold for deletion. It could also be explained by the use of six cores to represent the excised tumours, in the present study, rather than three cores in the previous studies. Our findings show a trend towards higher detection rate of 9p deletion with increasing number of scored cores (Figure 2). We showed a 100% concordance between whole-tissue sections and representative cores, with at least four out of the six cores scored, whether the tumour was 9p deleted or non-deleted.

It is important to note that we also applied the scoring technique suggested by La Rochelle *et al* (2010) using only the first three cores from each tumour on the TMA. The detection rate of 9p loss was 18% (15 out of 80), which matches the one reported by La Rochelle *et al* (2010)—14%. There was a significant difference in DSS at 10 years of follow-up between 9p-deleted and non-9p-deleted tumours in the same cohort of patients (20% vs 70%, respectively, $P < 0.001$ —Supplementary Figure 1) and 9p status based on La Rochelle's cut-off threshold remained an independent prognostic variable on multivariate analysis for DSS with T-stage, state of metastasis and sarcomatoid features (hazard ratio 2.956; $P = 0.025$).

Although homozygous deletion, hypermethylation of the promoter region and intragenic mutation have all been reported as mechanisms of *CDKN2A* inactivation in cell lines (Herman *et al*, 1995), they all seem to be rare events in primary renal tumours (Cairns *et al*, 1995; Sanz-Casla *et al*, 2003). Most of the studies looking at 9p deletion in primary renal tumours, and not in cell lines, found LOH of 9p21 locus (*CDKN2A*) to be the most commonly reported CNV (Schullerus *et al*, 1997; Grady *et al*, 2001; Presti *et al*, 2002; Brunelli *et al*, 2008; La Rochelle *et al*, 2010). In the present study, 91% (32 out of 35) of 9p deletion observed was due to the loss of one copy of the *CDKN2A* (either LOH or monosomy). Only three tumours showed deletion of both copies (homozygous deletion). It is well known that CNV correlates with gene expression (Knudson's theory)—loss of one allele allows the inactivation of the gene if the remaining copy is mutated or it could result in lower expression of the tumour suppressor protein. Aberrations, leading to low expression of p16INK4A and p14ARF, have been associated with aggressive ccRCC and poorer prognosis (Kawada *et al*, 2001b; Ikuerowo *et al*, 2007).

The principal findings of the present study are that ccRCC with 9p loss is associated with higher stage and larger size tumours, and

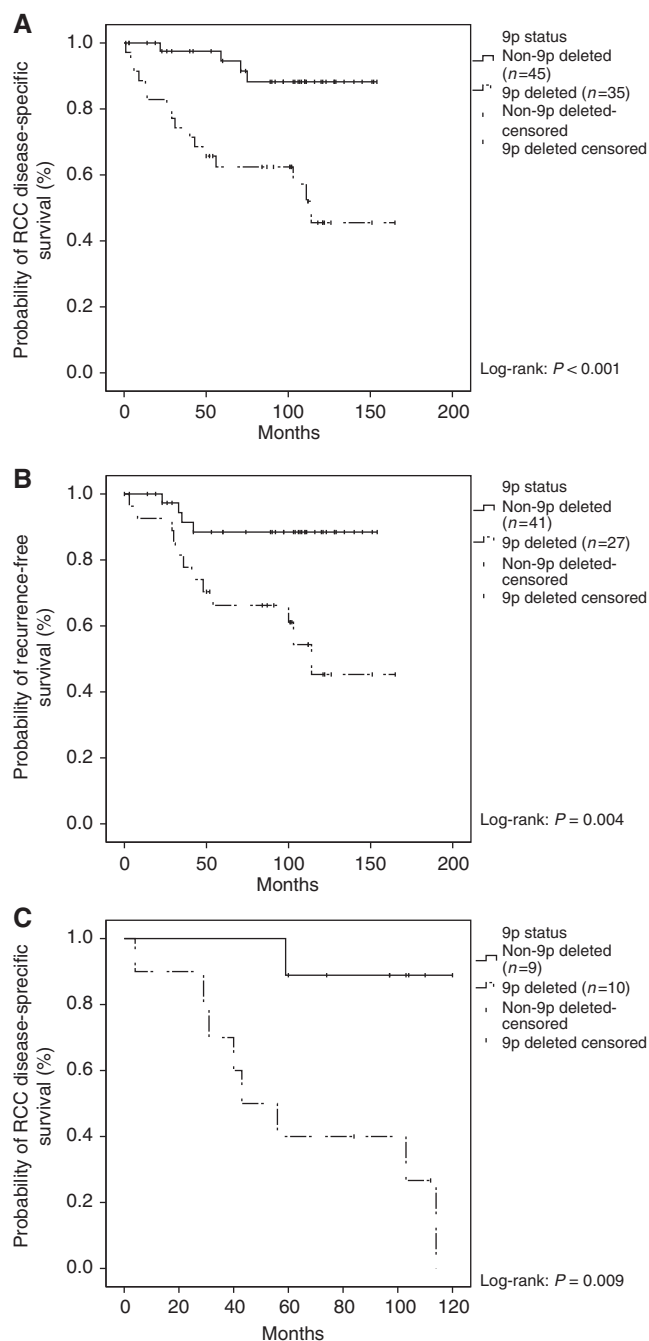


Figure 3. Kaplan–Meier survival analysis. **(A)** RCC disease-specific survival (DSS) by 9p deletion status. **(B)** Recurrence-free survival (RFS) in localised RCC by 9p deletion status. **(C)** Disease-specific survival (DSS) in locally advanced non-metastatic RCC (pT3N0M0) by 9p deletion status.

is more likely to have adverse histopathological features (tumour necrosis, microvascular invasion and renal vein involvement). A statistically nonsignificant trend was observed between 9p-deleted ccRCC tumours and higher Fuhrman grade. Our finding is in agreement with previous reports suggesting an association between 9p deletion and ccRCC progression (Klatte *et al*, 2009; La Rochelle *et al*, 2010; Dagher *et al*, 2013).

The prognostic impact of 9p deletion on ccRCC remains a controversial matter. Several studies assessed the impact of 9p status in ccRCC on survival using different molecular and genetic techniques with median follow-up periods ranging between 31 and 73 months. Two studies showed that 9p deletion was not associated

with poor prognosis (Presti *et al*, 2002; Antonelli *et al*, 2010). On the other hand, three studies concluded that 9p deletion was associated with worse prognosis on univariate analysis only (Moch *et al*, 1996; Schraml *et al*, 2001; Sanjmyatav *et al*, 2011). Whereas in two studies 9p loss remained an independent predictor of outcome in ccRCC on multivariate analysis. I-FISH was the main technique employed to assess 9p status in those two studies with different scoring techniques and threshold levels for deletion (Brunelli *et al*, 2008; La Rochelle *et al*, 2010). In our cohort of ccRCC, with the longest median follow-up of 95 months, survival analysis showed that patients with 9p-deleted tumours had a higher risk of recurrence and cancer-specific mortality. On multivariate analysis, it appears that 9p deletion remains a poor prognosticator for both outcomes, confirming the findings from previous studies based on I-FISH.

Several nomograms to predict the prognosis in RCC, relying on clinical and pathological parameters, have been developed and some have been externally validated. The integration of molecular or genetic biomarker, besides pathological and clinical parameters, has been attempted to improve the prognostication of some nomograms (Kim *et al*, 2005; Karakiewicz *et al*, 2007; Klatte *et al*, 2009). The addition of 9p status to prognostic models for ccRCC improved the predictive accuracy of DSS up to 89% (Klatte *et al*, 2009; La Rochelle *et al*, 2010). In the present study, SSIGN score and 9p status were independent predictors of outcome on multivariate analysis, mirroring the findings by Brunelli *et al* (2008). We have shown that the integration of 9p status to the well-established, externally validated, SSIGN score improved its predictive accuracy of DSS to 93.1%.

We acknowledge that the present study is limited by its retrospective nature. However, we made every effort to avoid bias. The use of preset scoring criteria, assessment of FISH score in a blinded manner by two independent observers, data collection using a validated electronic linkage methodology by third party, characterisation of tumours by a single pathologist with special interest in renal cancer, the representation of tumour by six cores on the TMA, use of normal tissue as a negative control to define threshold of 9p loss and systematic protocol-guided long-term follow-up exceeding those reported in the literature are the strengths of our study.

Lack of reporting of previous studies in a rigorous manner and published articles often showing insufficient information have been highlighted by reporting recommendations for a tumour marker prognostic studies (REMARK guidelines) (McShane *et al*, 2006) panel following a consensus meeting between US National Cancer Institute and the European Organisation for Research and Treatment of Cancer (NCI-EORTC) experts. We have adhered to these recommendations in reporting the present study (Supplementary Table 1).

The 9p deletion detected by I-FISH in ccRCC, regardless of the cut-off threshold, retained its prognostic value. Therefore, we believe that the potential use of this predictive biomarker in ccRCC should be further explored after agreeing on scoring technique, cut-off threshold for deletion and the number of cores required, keeping in mind intratumour heterogeneity.

We also suggest that a consensus statement is required to standardise I-FISH scoring for chromosomal deletion in renal tissue as this could have an implication on translational research in the area of renal cancer. 9p status could be used to predict behaviour of clinically localised SRMs and help in stratifying patients who may need interventions or surveillance. The change in size of SRMs on serial imaging is currently used as a surrogate marker of tumour progression in most of the centres across the world; however, this may not be the best indicator of aggressiveness. Our findings of worse prognosis in clinically localised SRMs (<4 cm) (Supplementary Figure 2) corroborates with previous reports and suggests that 9p-deleted SRMs could have a different behaviour and hence be a reason for early

Table 2. Univariate Cox proportional hazards analysis

				95% CI	
Variable	Categories	P-value	Hazard ratio	Lower	Upper
Univariate analysis for RCC DSS					
Age	Scale	0.074	1.036	0.997	1.076
Gender	Male vs female	0.758	0.867	0.035	2.149
Tumour size	Scale	<0.001	1.319	1.170	1.488
T-stage	pT3/4 vs pT1/2	<0.001	14.276	4.457	53.405
Metastasis	N + M + vs N0M0	<0.001	11.905	4.881	29.037
Necrosis	Present vs absent	0.009	3.235	1.338	7.821
Pelvic/lyceal invasion	Present vs absent	0.015	3.281	1.255	8.574
Microvascular invasion	Present vs absent	<0.001	5.397	2.148	13.560
Sarcomatoid features	Present vs absent	0.548	1.565	0.364	6.726
Fuhrman grade	G3/4 vs G1/2	0.042	3.121	1.043	9.340
9p status	Deletion vs no deletion	0.001	6.089	2.047	18.115
SSIGN score	5 Subcategories	<0.001	3.207	2.022	5.068
Univariate analysis for RFS					
Age	Scale	0.007	1.065	1.018	1.114
Gender	Male vs female	0.947	1.035	0.376	2.853
Tumour size	Scale	0.107	1.167	0.967	1.409
pT-stage	pT3/4 vs pT1/2	0.004	4.334	1.603	11.722
Necrosis	Present vs absent	0.160	2.026	0.757	5.417
Pelvic/lyceal invasion	Present vs absent	0.285	1.996	0.562	7.098
Microvascular invasion	Present vs absent	0.048	2.731	1.011	7.376
Sarcomatoid features	Present vs absent	0.213	2.584	0.581	11.502
Fuhrman grade	G3/4 vs G1/2	0.222	1.940	0.669	5.622
9p status	Deletion vs no deletion	0.008	4.642	1.494	14.421

Abbreviations: CI = confidence interval; DSS = disease-specific survival; RCC = renal cell carcinoma; RFS = recurrence-free survival; SSIGN = stage, size, grade and necrosis. Bold is used to highlight the statistically significant P-values.

Table 3. Multivariate Cox proportional hazards analysis

				95% CI	
Variable	Categories	P-value	Hazard ratio	Lower	Upper
Model 1 for RCC DSS					
9p status	Deletion vs no deletion	0.007	4.603	1.527	13.873
T-stage	pT3/4 vs pT1/2	0.002	8.329	2.181	31.803
Metastasis	N + M + vs N0M0	0.007	3.707	1.437	9.563
Model 2 for RCC DSS					
9p status	Deletion vs no deletion	0.014	4.002	1.322	12.111
SSIGN score	5 subcategories	<0.001	3.013	1.918	4.731
Model 3 for RFS					
T-stage	pT3/4 vs pT1/2	0.021	3.863	1.224	12.194
9p status	Deletion vs no deletion	0.021	3.323	1.198	9.220

Abbreviations: CI = confidence interval; DSS = disease-specific survival; RCC = renal cell carcinoma; RFS = recurrence-free survival; SSIGN = stage, size, grade and necrosis. Bold is used to highlight the statistically significant P-values.

intervention. Similarly, our observation of poor survival in 9p-deleted locally advanced non-metastatic (pT3N0M0) ccRCC, which was previously reported (Moch *et al*, 1996; Schraml *et al*, 2001), may be used to counsel patients for recruitment into adjuvant treatment for locally advanced tumours. This should, ideally, be further assessed in prospective randomised controlled trials.

CONCLUSION

Loss of 9p is associated with aggressive clear cell carcinoma of kidney and portends a worse prognosis in patients following surgery. Our findings independently confirm the findings of previous reports relying on I-FISH to detect 9p (*CDKN2A*) deletion.

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